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Non-Hodgkin's B cell lymphoma in persons with acquired immunodeficiency syndrome is associated with increased serum levels of IL10, or the IL10 promoter −592 C/C genotype

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Abstract

Interleukin-10 (IL10) may contribute to the development of non-Hodgkin's B cell lymphoma, especially in the context of acquired immunodeficiency syndrome (AIDS), where lymphoma incidence is greatly increased. Utilizing specimens from the Multicenter AIDS Cohort Study (MACS) obtained prior to diagnosis of AIDS-associated lymphoma, detectable serum human IL10 was seen much more frequently in lymphoma cases ($n = 61$, 26%) compared to CD4-matched AIDS controls (5%, $P = 0.004$), or to HIV-infected (2%, $P = 0.002$) or HIV uninfected subjects (0%, $P = 0.0003$). In longitudinal studies, detectable IL10 occurred at times closest to but preceding lymphoma diagnosis ($P = 0.01$). In an independent genetic analysis of single-nucleotide polymorphisms within the promoter region of the IL10 gene in 1157 MACS subjects, a high IL10-expressing genotype (−592 C/C) was overrepresented among lymphoma subjects ($P = 0.009$), even when controlling for race ($P = 0.006$). These results suggest that elevated serum IL10 or the IL10 promoter −592 C/C genotype are associated with development of AIDS lymphoma.

Keywords: Interleukin-10; B cell; Lymphoma; AIDS; Serum; Predictor; Single-nucleotide polymorphism; Genotype; Haplotype; Cytokine

Introduction

Little is known about immunologic changes and/or genetic markers that may be risk factors for the development of non-Hodgkin's B cell lymphoma, other than that the risk for developing these cancers is quite elevated in populations that have clinically apparent immune deficiencies [1]. In persons who are immunodeficient as a result of infection with human immunodeficiency virus (HIV), the risk of developing non-Hodgkin's lymphoma is 70 times greater than in the U.S. population overall [2]. Marked increases in B cell activation are commonly seen in persons with HIV

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infection or acquired immunodeficiency syndrome (AIDS), and are thought to contribute to the development of AIDS-associated B cell lymphoma (AIDS-lymphoma) [3–10]. In prior studies of serum samples obtained preceding a diagnosis of AIDS-lymphoma, we have observed significant increases in serum levels of immunological markers associated with B cell hyperactivation (soluble CD23, IgE, IL6, soluble CD27) in those subjects who went on to develop AIDS-lymphoma [11–15]. These and other B cell stimulatory factors could contribute to, or support the growth of, emerging malignant AIDS-lymphoma cells, by serving as autocrine/paracrine growth or viability factors. IL10 is such a B cell stimulatory cytokine (as reviewed in [16]), which is produced by AIDS-lymphoma tumor cells and/or surrounding cells in vivo, can act as an autocrine growth factor for AIDS-lymphoma cell lines in vitro, and is detectable in the serum of a significant proportion of patients after clinical recognition of AIDS-lymphoma [17–21]. However, no published information is available on whether increased serum IL10 levels precede AIDS-lymphoma, or if genetic polymorphisms demonstrated to increase IL10 gene expression are associated with the development of AIDS-lymphoma.

Defining in vivo levels of human IL10 in the circulation prior to the development of AIDS-lymphoma is one means of evaluating the role of this B cell stimulatory factor in lymphomagenesis. Early and/or sustained increases in serum levels of IL10 prior to clinical diagnosis may be contributing to the initial malignant transformation of B cells, while increased levels immediately preceding diagnosis may be the result of IL10 production by a nascent lymphoma. Independent of the levels of IL10 in the circulation at any given time, an individual's underlying genetic composition might also contribute to B cell lymphomagenesis by favoring continuously higher IL10 gene expression in the local environment of a potentially malignant B cell. It is known that within the upstream promoter region of the IL10 gene, there are three linked single-nucleotide polymorphisms (SNPs) at the −1082 (G/A), −819 (C/T), and −592 (C/A) positions [16], for which certain individual genotypes or combined haplotypes are associated with higher or lower promoter activity and IL10 production by peripheral blood cells following stimulation in vitro [22–24]. A high IL10-expressing promoter genotype (−592 C/C) has been previously evaluated in the context of HIV disease, and was shown to be associated with slower progression to AIDS [25]. However, this and other SNPs in the IL10 promoter region have not been examined in the context of AIDS-lymphoma.

We report here, for the first time, both immunologic and genetic evidence of increased IL10 prior to the clinical recognition of AIDS-associated B cell lymphoma. Utilizing archival sera obtained prior to lymphoma diagnosis, we show that detectable serum levels of IL10 are seen more frequently in men who go on to develop B cell lymphoma, compared to those who developed AIDS but not lymphoma, or to relatively healthy HIV-infected, or uninfected men. We also show, utilizing genotyping data from more than 1000 subjects, that non-Hodgkin's B cell lymphoma is more likely to occur in individuals with a high IL10-expressing promoter genotype.

Materials and methods

Study population

All study subjects are participants in the Multicenter AIDS Cohort Study (MACS). The MACS participants are homosexual men who have been followed at 6-month intervals in Los Angeles, Pittsburgh, Chicago, and Baltimore since the mid-1980s to examine the natural and treated histories of HIV infection and AIDS [26,27]. At each consecutively numbered visit, detailed histories and blood samples were obtained. Race/ethnicity data was collected by MACS participants' self-identification according to the indicated categories. National and local repositories of serum have been maintained. T cell enumeration on fresh specimens was determined by standardized flow cytometry at each visit. Clinical information from each visit, including AIDS-defining conditions and malignancies, were obtained from the participants and by review of external registries, and confirmed by medical record review. The protocols and questionnaires utilized in the MACS (available at www.statepi.jhsph.edu/macs/macs.html) have been approved by the Institutional Review Boards of the respective institutions.

For cross-sectional studies of serum IL10, frozen archived serum samples were obtained from HIV-infected MACS subjects who developed non-Hodgkin's B cell lymphoma, at the visit closest to but preceding lymphoma diagnosis (index visit), up to 36 months prior to lymphoma diagnosis. Serum samples meeting these criteria were available for 110 MACS lymphoma subjects. The index visit preceded the lymphoma diagnosis by 1–31 months (mean = 8 months), with 81% of subjects having an index visit that was not more than 12 months prior to lymphoma diagnosis.

Sera from three groups of nonlymphoma subjects were evaluated for serum IL10 in parallel with the lymphoma index visit sera. These consisted of samples from men with an AIDS diagnosis (according to the 1993 definition of the Centers for Disease Control and Prevention, excluding cases defined by CD4 T cell count alone) but no reported malignancy (AIDS, $n = 80$), HIV antibody-positive men who were relatively healthy (HIV+, absolute CD4 T cell counts >500 cell/mm^3, $n = 46$), and from HIV-negative men (HIV-, $n = 45$). These sera were selected from the same MACS visit number as the lymphoma index visit sera, to ensure consistent age and handling of frozen samples. A subset of lymphoma cases and AIDS controls ($n = 61$) were matched by visit number and absolute CD4 T cell numbers (±100 cells/mm^3); mean CD4 T cell counts for the matched

cases and controls were 133 and 137 cells/mm^3, respectively.

For longitudinal serum IL10 studies, archival serum samples were obtained for lymphoma subjects at the UCLA study site who had at least one additional sample available in the MACS repository at UCLA during the 2 years preceding the index visit. Lymphoma subjects were excluded if the index visit preceded the lymphoma diagnosis by more than 1 year. When available, additional samples were obtained at 2-year intervals preceding lymphoma diagnosis. Nineteen lymphoma subjects met these criteria, 11 subjects with detectable and eight subjects with undetectable serum IL10 on the index visit. The total number of visits per subject ranged from 2 to 8, with a time range of 1–104 months prior to lymphoma diagnosis. The two groups of lymphoma subjects (detectable vs undetectable IL10 on index visit) were not significantly different in mean time of index visit prior to lymphoma diagnosis (4 and 7 months, respectively), mean visit number tested (four visits), or mean time of earliest visit tested (48 and 47 months, respectively).

For genetic studies, all MACS subjects for whom IL10 promoter polymorphism data were available were included. This included a total of 1157 MACS subjects, 139 of whom were HIV-infected subjects with a non-Hodgkin's lymphoma diagnosis at any time during their participation in the MACS.

Serum IL10

Concentrations of human IL10 in sera were determined using a human IL10-specific enzyme-linked immunosorbent assay (ELISA) from Biosource International (Camarillo, CA), which has a lower limit of detection of 8 pg/ml. Although there are other commercially available ELISAs that claim greater sensitivity for the detection of IL10 in serum, they are either known to detect both human IL10 and Epstein-Barr Virus (EBV)-encoded viral IL10 (vIL10), or have not been evaluated for cross-reactivity with vIL10. EBV encodes a homologue of human IL10 that displays a high level of amino acid sequence homology to its human counterpart [16]. This high level of sequence homology results in the cross-detection of EBV vIL10 by most ELISAs that were developed to detect human IL10. Therefore, most commercially available IL10 ELISAs would provide results that would be ambiguous with regard to the relative contributions of human versus viral cytokine production. The Biosource human IL10 ELISA used in these studies has been documented by the manufacturer to be specific solely for human IL10, showing less than 0.2% cross-reactivity with the EBV vIL10. Assays were performed according to the manufacturer's instructions, with the exception of extending the initial sample incubation time to 3 h. Calculated concentrations of serum human IL10 were obtained using the KC Junior ELISA analysis software (BioTek, Inc., Winooski, VT).

IL10 genotype/haplotype analysis

Genotypes for SNPs at the −1082 and −592 nucleotide positions in the IL10 promoter region were available on 1089 MACS subjects, having been previously determined by Shin et al. using a polymerase chain reaction-restriction fragment length polymorphism assay [25] and augmented with additional genotyping using length-modified single-base extension [28]. An additional 68 MACS subjects had −592 SNP data only, for a total of 1157 subjects with −592 genotypes. A third IL10 promoter SNP is located at position −819, and has been shown to be in complete linkage disequilibrium with the −592 SNP (−819C/−592C; −819T/−592A) [22–25]. This was confirmed by genotyping at the −819 position in a subset of the MACS subjects included in this study ($n = 102$). IL10 promoter haplotypes, comprised of the combined SNPs at the −1082, −819, and −592 positions of the IL10 gene, were assigned based on −1082 and −592 data, plus −819 genotypes that were available from genotyping or inferred from the complete linkage disequilibrium observed between the −592 and −819 positions.

Statistical analyses

Comparisons of cross-sectional serum IL10 data from index visits of all lymphoma cases and AIDS, HIV+, or HIV− subjects were performed using a two-sided Fisher's exact test (detectable vs undetectable) or a bootstrap test for the difference of means (continuous levels of IL10). Serum IL10 data from lymphoma subjects and CD4-matched AIDS controls ($n = 61$) were compared using an exact McNemar's test for discordant pairs. Longitudinal serum IL10 data were analyzed by repeated measures logistic regression analysis. Comparisons of overall differences in the distribution of individual IL10 genotypes or IL10 promoter haplotypes in association with lymphoma status were made using exact chi-square analyses. Analyses of associations between pairs of factors (e.g., genotype or haplotype and lymphoma status) while controlling for others (e.g., race) utilized Cochran/Mantel-Haenszel chi-square statistics.

Results

Detectable serum human IL10 levels are seen more frequently preceding AIDS-lymphoma

Serum samples from the index visit (closest available visit preceding lymphoma diagnosis, not more than 36 months prior to lymphoma) were available from 110 MACS lymphoma subjects, as well as sera from 80 AIDS, 46 HIV+, and 45 HIV− subjects. Twenty-three of 110 men (21%) who developed AIDS-lymphoma showed detectable serum levels of IL10, a significantly higher frequency in comparison to the 4% detectable rate among AIDS subjects

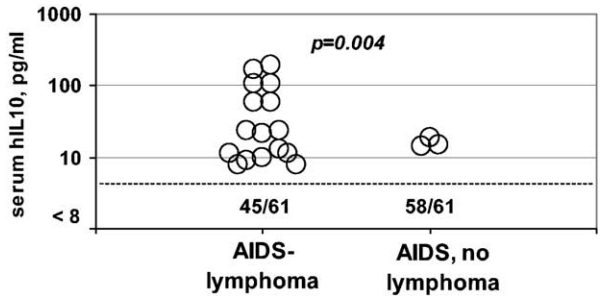

Fig. 1. Increased frequency of detectable serum IL10 preceding AIDS-lymphoma. IL10 levels in serum from the visit closest to, but preceding lymphoma diagnosis in subjects who go on to develop AIDS-lymphoma, compared to CD4-matched AIDS controls without lymphoma. The number of undetectable IL10 results (<8 pg/ml) obtained within each group is shown below the dotted line along the *x* -axis (number of undetectable IL10 values in group/61 total lymphoma cases or AIDS controls). *P* value shown is for the comparison of the frequency of detectable serum IL10 between the lymphoma cases and the AIDS controls.

($P = 0.0003$), 2% in HIV+ subjects ($P = 0.002$), or 0% in HIV− subjects ($P = 0.0003$). When lymphoma subjects were stratified by detectable vs nondetectable serum IL10, there was no significant difference between the two groups in the mean number of months between the index visit and the lymphoma diagnosis in the subjects (6 and 8 months, respectively; $P = 0.20$). There was no correlation seen between detectable serum IL10 and either absolute CD4 T cell number ($n = 108$, $P > 0.05$) or lymphoma subtype ($n = 47$, $P > 0.05$) among those lymphoma subjects for whom these data were available. When comparison of serum IL10 levels was restricted to those lymphoma cases and AIDS controls matched on absolute CD4 T cell number as an indicator of extent of HIV-induced immunodeficiency ($n = 61$), the difference in frequency of detectable serum IL10 (26% and 5%, respectively) remained highly significant ($P = 0.004$, Fig. 1). Among these CD4-matched cases and controls, lymphoma subjects had both increased frequency of detectable serum IL10 and higher levels of IL10 when detectable, with mean serum IL10 in the 16 lymphoma subjects who had detectable serum IL10 of 52 pg/ml (range = 8–196 pg/ml) compared to mean serum IL10 in the three AIDS controls who had detectable serum IL10 of 16 pg/ml (range = 14 to 19 pg/ml; $P = 0.001$).

Detectable serum IL10 is not a sporadic event preceding lymphoma diagnosis and is most likely to be seen close to diagnosis

In light of our observation that 20–25% of lymphoma subjects showed detectable serum IL10 at the index visit, we were interested in determining if detectable IL10 was associated temporally with the clinical diagnosis of lymphoma, or was a sporadic event that would be seen in 20%–25% of lymphoma cases at any time prior to lymphoma diagnosis. This was not possible to determine from the cross-sectional study, as the majority of index visit samples (81%) were obtained 1 year or less before diagnosis. To address the question, longitudinal serum samples were obtained from lymphoma cases from additional time points preceding the index visit, with at least one serum sample within 2 years prior to the index visit. Nineteen of the lymphoma cases met these criteria and were examined. Eleven of these cases had detectable serum IL10 on the index visit.

If detectable serum IL10 occurred sporadically among those who went on to develop lymphoma, then one would expect to see occasional detectable levels at time points other than the index visit in those subjects who had undetectable levels of serum IL10 on their index visit. This clearly was not the case, as IL10 was not detected in sera collected at any prelymphoma diagnosis time point from the eight lymphoma subjects who did not have detectable serum IL10 at the index visit (Fig. 2A). This included three subjects with serum samples collected more than 5 years prior to lymphoma diagnosis. In contrast, a significant percentage (64%) of lymphoma subjects who had detectable serum IL10 on the index visit also had detectable IL10 on at least one additional prior visit (Fig. 2B) ($P = 0.004$ compared to subjects in Fig. 2A). Once serum IL10 became detectable on a prelymphoma diagnosis visit, it tended to remain detectable on subsequent visits. Although three of the lymphoma subjects had detectable serum IL10 approximately 3 years prior to lymphoma diagnosis, a repeated measures logistic

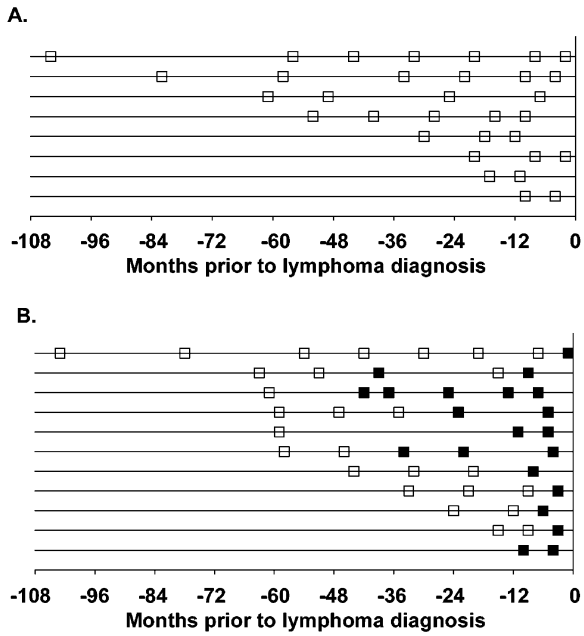

Fig. 2. Undetectable vs detectable serum IL10 in longitudinal sera from subjects who developed AIDS-lymphoma. Each horizontal line represents a single lymphoma subject over the months prior to lymphoma diagnosis, while each square along the line represents the serum IL10 result obtained on a particular visit. Open squares (□) indicate undetectable serum IL10 (<8 pg/ml); filled squares (■) indicate detectable serum IL10. (A) Lymphoma subjects with undetectable serum IL10 on index visit. (B) Lymphoma subjects with detectable serum IL10 on index visit.

Table 1
The C/C genotype of the IL10 promoter SNP at position −592 is overrepresented among subjects with AIDS-lymphoma

IL10 promoter −592 SNP	C/C	C/A	A/A
AIDS-lymphoma (n = 138)	66%	32%	2%
Without lymphoma (n = 1019)	54%	39%	7%
All genotyped MACS subjects (n = 1157)	55%	38%	7%
P value[a]		0.009	

[a] Comparison of genotype distribution between subjects with and without AIDS-lymphoma.

regression analysis including all 19 lymphoma subjects with longitudinal serum IL10 data suggested that detectable IL10 was most likely to be seen in the visits closest to the lymphoma diagnosis (P = 0.01).

An IL10 promoter genetic polymorphism that is associated with high expression of IL10 and slower progression to AIDS is also associated with the development of AIDS-lymphoma

Recently, it was reported that an IL10 promoter genotype that is associated with high IL10 expression (−592 C/C SNP) [22,29] is also associated with slower progression to AIDS over 5 years [25]. As IL10 can suppress the expression of HIV replication-enhancing proinflammatory cytokines, higher levels of IL10 might be viewed favorably in the context of HIV disease progression overall. However, IL10 is also a potent B cell stimulatory cytokine that has the potential to contribute to B cell hyperactivation and/or to the growth/viability of emerging lymphoma cells [16]. This led us to examine whether there might be an association between the occurrence of AIDS-lymphoma and IL10 promoter genotypes or haplotypes, especially those genetic types that have been shown to have high IL10 expression. Utilizing genotype data available on 1157 MACS subjects for IL10 promoter SNPs at the −592 and −1082 positions, we compared the distribution of genotypes and haplotypes between men who did and did not develop AIDS-lymphoma.

The −592 and −1082 IL10 promoter SNPs were first analyzed independently, stratifying subjects by lymphoma status and then comparing the distribution of subjects with each genotype. Significant differences in distribution were seen at the −592 position, with a greater proportion of lymphoma subjects having the homozygous −592 C/C genotype, and a corresponding reduction in the proportion of subjects with C/A and A/A genotypes compared to subjects without lymphoma (P = 0.009, Table 1). However, the −1082 genotype distributions were not significantly different among lymphoma and nonlymphoma subjects (24% and 19% G/G, respectively, P = 0.11).

Consistent with published data [22,23,25,30,31], our genotyped MACS subjects (84% of whom are white, non-Hispanic) showed a highly significant association between IL10 promoter genotype and race/ethnicity at both the −1082 and −592 positions ($P < 0.0001$). As expected, the highest frequency of the −592 C/C and −1082 G/G genotypes was seen among the white non-Hispanic (Caucasian) subjects, with white Hispanic (Latino) subjects having intermediate frequency, and black non-Hispanic (African–American) subjects having the lowest (−592 C/C, Fig. 3; −1082 G/G, data not shown). As shown in Fig. 3, among subjects with −592 genotyping, lymphoma frequency also varied significantly with race/ethnicity, as 121/971 (12%) of white, non-Hispanic and 13/70 (19%) of Hispanic subjects had a lymphoma diagnosis, while only 5/104 (5%) of black,

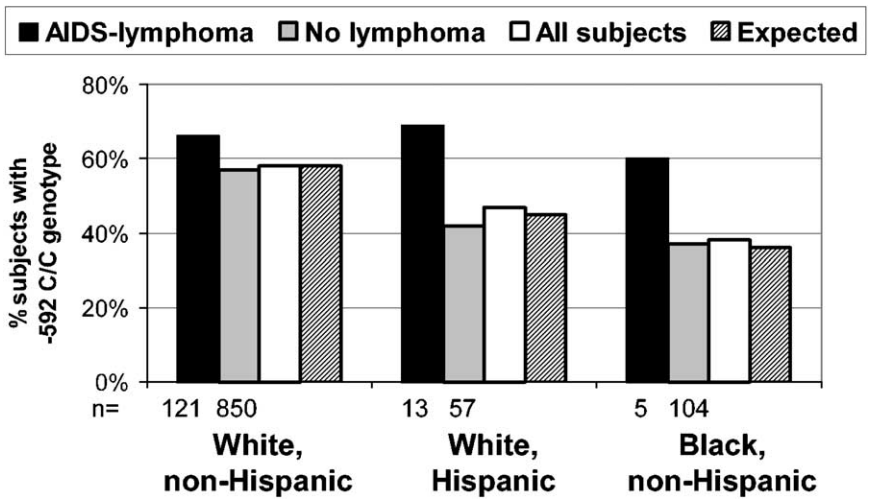

Fig. 3. The C/C genotype of the IL10 promoter SNP at position −592 is overrepresented among subjects with AIDS-lymphoma, even when taking race into account. “All subjects” are all genotyped MACS subjects of the indicated race; “Expected” is the genotype frequency for the indicated race as described in [23]. Number of subjects with and without lymphoma within each race group is indicated below the x -axis. P value for the overall association between lymphoma status and −592 genotype, taking race into account = 0.02.

Table 2
The IL10 promoter haplotypes predicted to have higher IL10 gene expression occur more frequently among subjects with AIDS-lymphoma

IL10 promoter haplotype (−1082, −819, −592/−1082, −819, −592) (predicted IL10 expression[a])	GCC/GCC (high)	GCC/ACC ACC/ACC (intermediate)	GCC/ATA ACC/ATA ATA/ATA (low)
AIDS-lymphoma (n = 124)	24%	42%	33%
Without lymphoma (n = 965)	19%	35%	46%
All haplotyped MACS subjects (n = 1089)	20%	35%	45%
P value[b]		0.02	

[a] Predicted IL10 gene expression as described by Shin et al, [25], where low expressors are defined by one or two A alleles at position −592; high expressors are homozygous non-A at both −592 and −1082 positions.
[b] Comparison of distribution of high, intermediate, and low IL10-expressing haplotypes between subjects with and without AIDS-lymphoma.

non-Hispanic subjects had lymphoma (P = 0.022). There were no lymphoma cases in seven genotyped subjects of other races/ethnicities, who were excluded from further analyses involving race.

When IL10 promoter genotype, race/ethnicity, and lymphoma were taken into account in a single analysis for each SNP, lymphoma was still significantly associated with the C/C genotype at the −592 SNP (P = 0.02), and not associated with the G/G genotype at the −1082 SNP (P = 0.22, data not shown). The overrepresentation of the C/C genotype at −592 in association with lymphoma was particularly striking in Hispanics and Blacks, where the frequency of C/C among lymphoma subjects was still at or above 60%, even though the expected frequencies in these two groups are 45% and 36%, respectively (Fig. 3) [25]. An increased risk of lymphoma could be demonstrated in subjects with the C/C −592 SNP (OR = 1.6, 95% CI 1.1–2.3) or the G/G −1082 SNP (OR = 1.3, 95% CI 0.8–2.0), when compared to those subjects with intermediate/low IL10-expressing genotypes, controlling for race. However, it became apparent that any increased lymphoma risk associated with the −1082 SNP was due solely to its genetic linkage to the SNP at −592, as there was no independent association between the −1082 SNP and lymphoma when taking the −592 SNP into account at the same time (P = 0.9).

Utilizing 1089 of the MACS subjects for whom genotyping data was available at positions −1082 and −592, and for whom genotyping data at position −819 was either available or could be inferred, we compared the distribution of the IL10 promoter haplotypes among subjects with and without lymphoma, taking race into account in all analyses. When considering all six IL10 promoter haplotypes, the overall association between lymphoma and haplotypes showed borderline significance (P = 0.049, data not shown). Because the six haplotypes have been associated with different levels of IL10 expression in vitro, additional analyses were performed by stratifying the haplotypes according to expected levels of IL10 expression.

There is a clear consensus in the literature that the GCC/GCC IL10 promoter haplotype (−1082 G/G, −819 C/C, and −592 C/C) is associated with the highest levels of IL10 expression, while the ATA/ATA haplotype (−1082 A/A, −819 T/T, and −592 A/A) is associated with the lowest levels of IL10 expression [22–24,32]. However, there are conflicting approaches to characterizing the level of IL10 expression associated with the other four haplotypes that include at least one heterozygous SNP [23–25,32]. Therefore, we used three different approaches for stratifying IL10 promoter haplotypes into high, intermediate, or low expected levels of IL10 expression, based on these published reports, then analyzed each for associations between expected IL10 expression and lymphoma.

In the first two analyses, we stratified the IL10 promoter haplotypes according to −592 genotype (as shown in Table 1), ignoring the −1082 genotype [29], and conversely, according to −1082 genotype, ignoring the −592 genotype [23,24,32]. In a third approach, we used a −592 A dominant (low) model described by Shin et al. when considering IL10 promoter genotypes in the context of HIV disease progression [25]. In this model, low IL10 expression is predicted for any IL10 promoter haplotype bearing A at −592 (A/A or A/C), while haplotypes containing homozygous C/C at −592 are predicted to have intermediate IL10 expression when combined with heterozygous G/A at −1082, and high IL10 expression when combined with homozygous G/G at −1082.

As might be expected based on the analyses of −592 and −1082 genotypes independently, IL10 promoter haplotype stratification based on −1082 genotypes alone failed to show any significant association between expected IL10 expression and lymphoma overall (P = 0.12), while stratification based on −592 genotypes alone showed a highly significant association between the high IL10-expressing haplotype (GCC/GCC) and lymphoma (P = 0.006). Stratification of IL10 haplotypes utilizing the −592 A dominant (low) model, which takes both −592 and −1082 genotypes into account, also showed a significant association between predicted levels of IL10 expression and lymphoma overall (Table 2, P = 0.02), but not as strong as that seen when the −1082 genotypes were completely disregarded. In both the −592 alone and the −592 dominant A (low) analyses, the higher IL10-expressing haplotypes were more highly represented among subjects with lymphoma, with a clear re-

Table 3
IL10 promoter genotype and observed serum IL10 levels appear to be independent of one another in subjects who go on to develop AIDS-lymphoma

IL10 promoter −592 SNP	C/C	C/A	A/A
AIDS-lymphoma, detectable serum IL10 ($n = 21$)	71%	29%	0%
AIDS-lymphoma, undetectable serum IL10 ($n = 79$)	63%	34%	3%
P value[a]		0.8	

[a] Comparison of distribution of IL10 promoter −592 SNP between lymphoma subjects with and without detectable serum IL10 in the visit closest to but preceding AIDS-lymphoma diagnosis.

duction in the proportion of lymphoma subjects with low IL10-expressing haplotypes.

Expected IL10 gene expression and detectable serum IL10 appear to be independent indicators preceding AIDS-lymphoma

Characterization of IL10 promoter genotype/haplotypes as high, intermediate, or low IL10-expressors is based on promoter activity assays and/or the production of IL10 by cells following various types of stimulation in vitro [22–25,29,32]. In these previous studies by others, there were no data available on serum IL10 levels in vivo to which the in vitro IL10 expression could be compared. We were interested, therefore, in whether expected IL10 expression (based on IL10 promoter genotype or haplotype) was consistent with our observed serum IL10 levels.

Among the 43 subjects without lymphoma for whom both IL10 promoter genotype and serum IL10 data were available, no detectable serum hIL10 was seen regardless of IL10 promoter genotype/haplotype. Among 100 subjects with lymphoma for whom both genotype/haplotype and serum data were available, 21 had detectable serum hIL10 in the index visit sample. While the frequency of the −592 C/C IL10 promoter SNP was slightly higher among the lymphoma subjects who had detectable serum IL10 (Table 3), both groups of lymphoma subjects had similar overall distributions of C/C vs C/A vs A/A at the −592 position ($P = 0.8$). Likewise, while the two highest serum IL10 levels were seen in lymphoma subjects with the −592 C/C genotype, no consistent association was seen between −592 genotype and measured level of IL10 among the 21 lymphoma cases with detectable serum IL10 ($P = 0.5$, data not shown).

Discussion

IL10 is a potent stimulator of human B cells [16] that is produced in situ by AIDS-associated B cell lymphoma tumor cells, and has been demonstrated to act as an autocrine growth factor in AIDS-lymphoma [16–20]. Detectable and/or increased serum levels of IL10 have been reported to be associated with AIDS-lymphoma at, or following, clinical recognition of these tumors [21,33], leading to the suggestion that, at least in a proportion of AIDS-lymphoma cases, detectable serum IL10 reflects the presence of cytokine-secreting tumor cells [21]. What has not been previously explored, however, is the potential role of increased levels of IL10 prior to the clinical recognition of lymphoma, as an indicator of a nascent lymphoma, and/or as a contributing factor to the initiation and development of B cell lymphoma, particularly in persons with a genetic background associated with high IL10 expression. In the studies described here, we report that the development of AIDS-lymphoma is associated with increased levels of serum IL10 prior to diagnosis of lymphoma, and independently, with a genetic IL10 promoter haplotype associated with high IL10 production.

In previous work, we have shown that there are other B cell stimulatory molecules that are elevated in the serum preceding the development of AIDS-lymphoma [11–15]. In a cohort study like the MACS, where there are large numbers of well-characterized HIV-infected subjects and a repository of archived sera collected over time, we have the unique opportunity to evaluate circulating levels of IL10 in vivo using serum samples available from time points prior to the diagnosis of lymphoma. In a cross-sectional study using the closest available serum sample preceding lymphoma diagnosis (index visit), detectable serum human IL10 was five times more likely to be seen in men who went on to develop AIDS-associated lymphoma compared to CD4-matched controls with AIDS but not any type of malignancy (26% vs 5%, Fig. 1); little or no detectable serum IL10 was seen in relatively healthy HIV+ (2%) or HIV-uninfected (0%) subjects. In addition, when serum IL10 was detectable preceding a lymphoma case, it was often at levels much higher than those seen in the rare nonlymphoma subject with detectable IL10. These results demonstrate that, although the human IL10-specific ELISA chosen for this study has a higher cutoff for level of detection than other ELISAs that have been used to evaluate serum IL10 in HIV-infected individuals [21,33–35], it was clearly specific and sensitive enough to detect real differences between subjects who did and did not develop AIDS-lymphoma. In a longitudinal study utilizing a subset of the lymphoma subjects, it was clear that detectable IL10 prior to diagnosis was not a sporadic event (Fig. 2), and was most likely in the months immediately preceding a clinical diagnosis of lymphoma. These data support the hypothesis that IL10 may indeed be the product of a nascent tumor [21] and/or of tumor-responsive cells such as macrophages in the local environment [19]. They also demonstrate that in a small but significant proportion of individuals who are in the process of developing a clinically apparent lymphoma, IL10 is made

in sufficient amounts to cross the threshold of detection prior to diagnosis.

Because there was no correlation seen between serum IL10 and histological subtype of the subsequent tumor or extent of HIV disease (as indicated by absolute CD4 number), detectable serum IL10 appears to be independent of these factors. This raises the possibility that serum IL10 level, especially if clearly elevated above the level of detection, could be useful as a predictor in HIV-infected men to identify those who might have a nascent tumor and/or be at high risk of a lymphoma diagnosis in the relatively near future. The fact that only approximately one-quarter of lymphoma subjects show detectable IL10 levels prior to diagnosis limits the predictive power of serum IL10 on its own. However, the development of new, more sensitive assays that are specific for human IL10 may ultimately allow the detection of serum IL10 in a greater fraction of developing AIDS-lymphoma cases. Although IL10 was detectable in the serum of only a minority of lymphoma cases, it was very rarely detected in the serum of nonlymphoma subjects. This suggests that detectable serum IL10 in combination with other markers of B cell activation that are known to be elevated prior to AIDS-lymphoma might be a powerful predictor of B cell lymphoma. In a preliminary analysis examining serum IL10 and IL6 data from 47 of the MACS lymphoma subjects included in this report, detectable IL10 was more likely among men with detectable IL6 compared to men with undetectable IL6 (46% vs 21%, respectively), but the difference was not significant ($P = 0.14$). However, when these 47 lymphoma subjects were compared to 44 matched AIDS control subjects, the combination of detectable IL6 and detectable IL10 was only seen in lymphoma subjects (13% vs 0%, $P = 0.03$), suggesting that combinations of markers detected in the serum prior to lymphoma may indeed help to identify some persons at risk for lymphoma. Utilizing our MACS serum data on IL10 and IL6, as well as additional markers of B cell activation and differentiation, including immunoglobulins, soluble CD23 (sCD23), sCD27, sCD30, and sCD44, we have found no strong correlation between serum IL10 and any other single marker, indicating that IL10 is an independent immunologic marker of AIDS-lymphoma [11–15, unpublished data]. However, we are developing a multifactorial model that combines serum IL10 with other markers that may be able to predict an increased risk for the development of AIDS-lymphoma [E.C. Breen, W.J. Boscardin, H. Guo, R. Detels, and O. Martínez-Maza, manuscript in preparation].

While detectable serum levels of IL10 may reflect cytokine production by an established but as yet undetected tumor, increased but localized IL10 production that results in circulating levels below the threshold of detection may be important in earlier stages of B cell lymphomagenesis. IL10 gene expression and production of this type are likely to be influenced by the underlying genetic makeup of each individual, particularly through genetic polymorphisms such as SNPs in critical promoter and/or coding regions of the IL10 gene. There is a growing body of work linking genetic polymorphisms associated with increased expression of B cell stimulatory cytokine and/or cytokine receptor genes other than IL10 to the development of AIDS-associated malignancies [36–40]. This suggested to us that there might also be a link between genetic polymorphisms favoring higher IL10 expression (such as the IL10 promoter SNPs −1082 G/G and the −592 C/C) and the development of AIDS-lymphoma. While these IL10 promoter SNPs have previously been examined in the context of progression to AIDS overall [25], they had not been examined specifically in the context of AIDS-associated B cell lymphoma.

When we evaluated IL10 genotyping data from over 1000 MACS subjects, we observed that an IL10 promoter genotype (Table 1) and haplotypes (Table 2) associated with higher IL10-expression were overrepresented among men who developed AIDS-lymphoma, even when controlling for differences in genotype frequencies according to race/ethnicity (Fig. 3). The association between lymphoma and IL10 promoter genetic type appeared to depend predominantly, or perhaps even exclusively, on the genotype at the −592 SNP, as significant associations between expected IL10 expression and lymphoma were seen only when IL10 haplotypes were stratified according to −592 genotype alone or by a −592 A dominant model ($P \leq 0.02$, Table 2). Interestingly, our observation of a higher frequency of the −592 C/C promoter genotype among subjects with AIDS-lymphoma parallels the well-documented observation of higher frequencies of the same genotype among whites and Hispanics, which in turn parallels our observation of higher frequencies of AIDS-lymphoma among these two race groups compared to blacks within the MACS subjects studied (Fig. 3). This raises the possibility that the IL10 genetic background may be a direct contributor to the genesis of AIDS-lymphoma, and/or tightly linked to one or more other causative genes that give rise to the differences in lymphoma incidence among racial/ethnic groups.

Our observation of an association between the −592 C/C IL10 promoter genotype and an unfavorable clinical outcome, i.e., AIDS-lymphoma, may at first glance appear to be contradictory to the results of Shin et al, where the same genotype was associated with a favorable clinical outcome, i.e., reduced risk of progression to AIDS after five years [25]. Shin et al. hypothesized that their observed reduced risk for progression to AIDS was the result of greater anti-inflammatory activity due to increased levels of IL10, which decreased production of inflammatory cytokines, and resulted in reduced HIV replication. However, IL10 is a pleiotropic cytokine, which in addition to having anti-inflammatory properties, has the ability to directly stimulate B cells and to indirectly inhibit T cell activation through its action on antigen-presenting cells [16]. While its anti-inflammatory properties may help to slow the progression to AIDS in an HIV-infected population overall, our results suggest that in the subpopulation of HIV-infected individ-

uals who go on to develop B cell lymphoma, higher IL10 expression may be conducive to lymphomagenesis and/or tumor cell growth, by driving B cell activation and/or by inhibiting T cell responses that might otherwise keep newly malignant B cells under control. Among the MACS subjects genotyped for the −592 IL10 promoter SNP (most of whom were also included in the Shin study), only 12% developed lymphoma (Table 1). It seems likely, therefore, that the detrimental effect of the high IL10-expressing −592 C/C genotype (lymphoma) in such a small minority of subjects was obscured by the apparent beneficial effect (lower risk of progression to AIDS) in the much larger majority of subjects who never developed lymphoma.

Just as no single cytokine is likely to be acting in isolation in vivo, there may be a combination of polymorphisms within immunologically relevant genes that play a role in the development of AIDS-lymphoma. Two other genetic polymorphisms have already been identified in chemokine and chemokine receptor genes that are associated with AIDS-lymphoma. In two studies of a B cell stimulatory cytokine known as stromal cell-derived factor 1 (SDF-1, also called pre-B cell-stimulating factor), a SNP in the 3′ untranslated region (SDF-1 3′UTR 801A/A or A/G) that is associated with increased levels of SDF-1 expression, was seen to be associated with an increased risk for AIDS-lymphoma [39,40]. In addition, AIDS-lymphoma in children was preceded by increased levels of SDF-1 mRNA [40]. In studies by MACS investigators [36], and others [39], it was shown that being heterozygous for a mutant, truncated form of the CCR5 chemokine receptor (CCR5 Δ32) was associated with a markedly decreased risk for the development of AIDS-lymphoma. Because CCR5 is the receptor for RANTES, MIP-1α and MIP-1β, cytokines that have B cell stimulatory properties, it was postulated that the decreased risk for AIDS-lymphoma seen in CCR5 Δ32 heterozygotes was due to a lower level of RANTES-induced B cell activation in these subjects [36]. Our observations of an increased risk of AIDS-lymphoma in persons with high IL10-expressor promoter haplotypes or with increased circulating levels of IL10 are consistent with these reports, all of which suggest that the presence of higher levels of B cell stimulatory cytokines, or of a functional receptor for such cytokines, may contribute to an environment conducive to AIDS lymphomagenesis. Additional studies are planned or are underway to determine and/or analyze multiple genetic polymorphisms among subjects in the MACS who developed lymphoma at these and other loci, including the CCR5-binding chemokines and IL6 [37,38].

There is very limited and contradictory information available in the literature regarding the correlation (or lack thereof) between IL10 promoter haplotypes and measurements of circulating levels of IL10 [41–43]. These studies have reported no correlation between IL10 promoter haplotype and serum IL10 levels [41], or higher serum IL10 levels in association with either the low IL10-expressing haplotype (ATA/ATA) [42] or the high IL10-expressing genotype at the −1082 position (G/G) [43]. In all of these studies, the specificity of the ELISA used for human IL10 vs EBV vIL10 was not addressed, raising the possibility that the low levels of IL10 reported (< 5 pg/ml in the majority of subjects) and/or the small serum IL10 differences between groups may have been of viral (EBV) origin, and so were unrelated to subjects' underlying IL10 promoter genotypes. In our study, which measured only the contribution from the human IL10 gene, while both detectable serum IL10 and the high IL10-expressing −592 C/C genotype were more likely among lymphoma subjects, there was no clear association between the two (Table 3). In light of our longitudinal data that suggests detectable serum IL10 may be the result of cytokine secretion by a developing tumor or its local environment, it is not surprising that differences in IL10 expression attributable to underlying genetic polymorphisms in the IL10 gene may be too subtle to be detected in serum. This does not rule out, however, the potential contribution of an individual's IL10 promoter haplotype to B cell lymphomagenesis by driving (relatively) increased levels of IL10 expression on a continuous basis, perhaps in a very localized manner that precludes detection in the circulation in general, but supports the initial transformation and/or survival of malignant B cells. Once a tumor has been established, its own production of IL10 may then provide a valuable means of detecting its presence by sampling the serum. Based on our results in an HIV-infected population at greatly increased risk for B cell lymphoma, IL10 genotype and serum levels should be viewed as being independently associated with the development of AIDS-lymphoma, and in concert with other cytokines and cytokine genetic polymorphisms, may help to better define the genetic and immunologic factors that contribute to the development of B cell lymphoma in the context of immunodeficiency.

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